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The primary objective of this study was to help to establish the aquatic bioassay as a valid alternative to rodent carcinogenicity and toxicity testing for use by the U.S. Army to test contaminated ground water and effluents in a rapid, inexpensive manner. The study had two parts: the first to study normal hepatic parenchymal development in the fish species to be used, the medaka, and the second to perform a controlled carcinogenic exposure study using the medaka with documentation of the lesions and neoplasms produced. The results outlined in this report have shown that the protocol used is potentially a rapid method of testing carcinogens using the medaka as a model.

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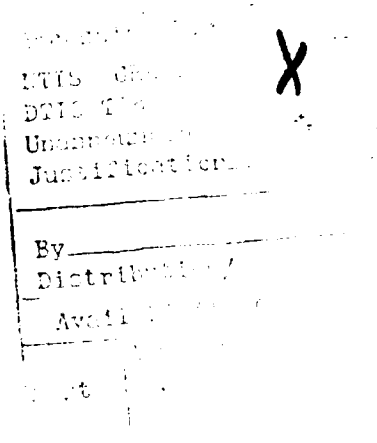

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I. Introduction

A. Objectives and Significance

This proposal had two major objectives related to the development of an aquatic bioassay for carcinogenicity and toxicity testing using the medaka. The first was to study the normal development of the liver of the medaka including morphologic and morphometric evaluation of the hepatic tissue. The rationale was to use this database to help make informed diagnosis and interpretations of cellular changes in some of the proliferative and neoplastic lesions produced by carcinogenic exposure. The second was to perform a controlled study in which the medaka was exposed to a known carcinogen, diethylnitrosamine, in order to determine the sequential development and progression of certain lesions to neoplasia, and to assess the significance that these lesions have on the host. The value of this work is that the information obtained can be applied to the performance and interpretation of aquatic bioassays by the United States Army to test complex environmental mixtures such as effluents and contaminated ground water in a rapid, and relatively inexpensive manner.

B. Background

Environmental contamination with various carcinogenic and toxic compounds has led to massive research efforts into determining human risk involved in exposure to these substances. The use of animal models to study this risk has come about because known carcinogens for man are also carcinogenic for animals, and the morphology and behavior of certain types of carcinogen induced neoplasms in man are similar to those in animals (1).

One important source of exposure is waterborne contamination, as water in the form of rain, lakes, and oceans is a tremendous disseminator of substances. The level of environmental pollution in the United States is great, and an effect on fish has been evidenced by a high prevalence of neoplasms in certain fish in various locations around the U.S. (2). This observation has strongly suggested the possibility of using small fish species as alternatives to small mammals in carcinogenicity and toxicity testing bioassay systems. The use of small fish bioassays is attractive because of the reduced time requirement for testing compared to rodent studies which can take up to 2.5 years, the ability to use large numbers of small fish, and the decreased expense of maintaining the animals and administering the test substances. With an aquatic test system which includes proper control of light, temperature, and salinity large numbers of compounds can be tested with relative ease. A short reproductive cycle also allows assessment of effects on embryonic immature and adult stages of life.

The medaka (Oryzias latipes) is a small (3.0-3.5 cm long) aquarium fish native to Japan, and has been considered attractive for carcinogenesis and toxicity testing for the following reasons: 1) large numbers can be utilized in a small space, 2) it is a hardy fish and can be easily maintained and bred, 3) males and females are easily distinguished by external features, and 4) it is highly sensitive to known carcinogens (3). The exposure of the young adult (4-5 month old) medaka to diethylnitrosamine (DEN) in concentrations of 15, 45, and 135 ppm for 8 weeks produced hepatomas in all survivors in the mid and high dose groups, and 3/16 in the low dose group by 13 weeks (4). Shorter exposures produced degenerative hepatocellular changes of hyalin granules, increased basophilia, and cellular swel-

ling (5). Bizarre basophilic proliferative foci appeared after 4-5 week exposures. The exposure of medaka embryos to DEN for 10 days at 25, 50, or 100 ppm produced hepatic tumors beginning at 3 months of age. Basophilic foci sometimes containing mitotic figures were also described (6). Other studies have produced similar results (7,8).

The liver is an organ essential to maintaining life and is diverse both functionally and cellularly. It stores, secretes, synthesizes, metabolizes, and detoxifies various substances. The main cell populations of the mammalian liver include hepatocytes, bile duct epithelium, Kupffer cells, endothelial cells, Ito cells, and pit cells. The arrangement of hepatocytes in the fish liver is different from the lobular pattern seen in mammals, and is described as a tubulosinusoidal pattern with indistinct lobulation (9), and a characteristic two cell-thick cell plate. The ultrastructural composition of the teleost hepatocyte has been reviewed (10,11) and is basically similar to rat and man, although there are differences (as outlined below). It is a polygonal cell with a centrally located round nucleus, smooth (SER) and rough endoplasmic reticulum (RER), mitochondria, lysosomes, glycogen, and lipid vacuoles. Peroxisomes can be observed. Bile canaliculi are formed as modifications of the hepatocyte membrane into microvillus processes, and are connected by junctional complexes. Bile duct epithelium has a lumenal orientation, small numbers of mitochondria, rough ER, and occasional lysosomes, and is limited by a basal lamina (12). Structures present in the fish liver but not seen in human or rat are the melanomacrophage centers, and islands of exocrine pancreas.

There has been considerable debate over the existence of Kupffer cells, or stationary macrophages, in fish liver, but they are considered to exist in small numbers in some species (11). They are located side by side with endothelial cells, and contain abundant rough endoplasmic reticulum (ER), lysosomes, and phagosomes (12). The Ito cell is presumed to be a primitive mesenchymal type cell located in the perisinusoidal space that may differentiate into fibroblasts and/or fat cells (13,14). In goldfish, they are characterized by cytoplasmic microfilaments and maintain desmosomal attachments with hepatocytes, endothelial cells, and each other (15). The pit cell is a cell of unknown function found in various locations in the teleost liver. It contains characteristic dense core granules and may have an endocrine function (16).

Any of these hepatic cell populations may be altered in morphology and/or distribution by exposure to carcinogens, and Part B of the present proposal will identify and describe the various populations in the normal adult medaka liver so that comparisons with transformed cells can be made.

Developmentally, the mammalian liver arises as a bud from the endodermally derived foregut, and the parenchymal cells, intrahepatic biliary tree, and endothelial cells are of endodermal origin. Kupffer cells, fibrous, and hematopoietic tissues are thought to be derived from the splanchnic mesoderm of the septum transversum (17). The Ito cells may also be of mesodermal origin, and the origin of pit cells is unknown. Embryogenesis in the medaka lasts 11-12 days, at which time hatching occurs (6). The developmental stages of a small esturine fish, Rivulus

marmoratus, have been described, and as hatching occurs at 12-13 days, the stages may be generally applied to the medaka. The liver bud appears at 90 hours post fertilization at Stage 26 of development in Rivulus (18). Reports of subsequent hepatic development in fish are few and are often related to the reproductive cycle and vitellogenesis.

II. Specific Aims

Part A. For the small fish bioassay for toxicity and carcinogenicity testing to become valid, documentation of the fish tissue responses to carcinogens had to be made. To accomplish this goal, the medaka was exposed to low, medium, and high levels of the known carcinogen, diethylnitrosamine (DEN). Degenerative, proliferative and/or neoplastic foci in the liver were documented and compared to the sequential hyperplastic and neoplastic events which have been well documented in the rat. Selected specimens were also assessed ultrastructurally to make cellular identifications and to define certain alterations. In addition, autoradiography was employed to assess the degree of proliferative activity not only of these hepatocellular foci, but also of other hepatic cell types which may be affected and which will have been categorized as described in Part B.

Part B.

Using morphologic and morphometric technique, the sequential development of various hepatic cell types in the medaka were documented to establish baseline criteria for accurate identification of tumors that may develop due to chemical exposure. Comparisons to mammalian hepatocellular development and differentiation were also made and similarities and/or differences determined.

III. Body of Work

A. Methods

For Part A, medaka (Oryzias latipes) were raised in accordance to established standards at the United States Army Biomedical Research and Development Laboratory at Fort Detrick, Maryland. At the age of 14 days post-hatching, groups of male and female medaka were randomly separated into four groups (I-IV) and exposed to 100 mg/L (I), 200 mg/L (II), 400 mg/L (III), or 0 mg/L (IV) of DEN for 48 hours by addition to the tank water, and then removed to clean water for periods of up to six months. Subsets of approximately 10 fish from each group were killed at approximately 14, 30, 45, 60, and 90 days post-exposure, and all remaining fish killed at 180 days post-exposure. Livers were examined for gross lesions and placed in Bouins fixative and processed for histology in glycol methacrylate, with additional small sections of liver placed in 4.0% cold glutaraldehyde and processed for electron microscopy in Araldite.

For autoradiography using the mid-dose group, the number of fish in parenthesis (Table I) were separated out and injected intraperitoneally with tritiated thymidine [^{3}H]TdR (New England Nuclear, Boston, MA, 1mCi/ml; specific activity 6.7 Ci/mmol) at a dosage of 10 uCi/fish two hours prior to sacrifice. Paraffin embedded 3 u sections of liver were dipped in Ilford L4 emulsion to create a monolayer of halide crystals over the slide, and the slides sealed in light tight boxes for 3-4 weeks. Slides were then radiographically developed and stained with hematoxylin and eosin. Unfortunately, at any sacrifice only minimal labeling was seen; occasionally in skin epithelium or thyroid. Liver did not label at anytime and reasons for this will be discussed in the Conclusion section.

For Part B, medaka embryos and fry were collected and processed as per the original protocol. Fish were separated into three groups; < 11 days post hatch, 32 days post hatch, and 61 days post hatch for morphometric evaluation and comparison. Micrographs were taken from each liver and all enlarged to 5000x. The volume fraction (percentage) of each compartment assessed was determined according to the following formula Px/P_t where Px equals the points falling over component of interest compared to the total points (P_t) using a 378 point grid overlay on each micrograph. Results were analyzed using the BMDP statistical package for comparisons between groups.

B. Results - Part A

The results of this portion of the study resulted in 2 presentations and 3 publications (20,21,22) which are included on a separate page. An additional manuscript was also produced regarding this work for the annual United States Army Biomedical Research and Development Laboratory workshop, 1989. The overall results are summarized here.

There was a direct relationship between the exposure level of DEN and the incidence and severity of many of the lesions observed. This was particularly true in comparisons between the low level exposure group and the two higher exposure levels as the latter two groups exhibited many similarities. However, the highest exposure level produced the most profoundly affected livers. In group I (100 mg/L DEN) lesions often involved a minimal portion of the liver and more fish throughout the study maintained histologically normal appearing livers. No hepatic malignancies were observed in this group within the time frame of this study. By contrast, in group III (400 mg/L DEN) lesions were often complex and extensive and only few

fish at any time during the study had histologically normal livers. Several hepatic malignant tumors were seen in this group. Changes in group II (200 mg/L DEN) fell somewhere in between groups I and III. Table I shows the percentages of medaka at each sacrifice which exhibited a given change. Although somewhat misleading because the severity of the change is not indicated, differences between groups can be appreciated. In controls taken at each sacrifice, none of the changes described below were seen except for minimal spongiosis hepatis in a few fish from the final sacrifice.

The progression of changes seen is shown in Table II, and was characterized by early onset of hepatocellular degeneration and necrosis followed by a variety of proliferative and neoplastic changes. At 14 and 31 days post-exposure (PE), degenerative changes were characterized by individual hepatocellular necrosis (IHN) recognized histologically by marked cellular swelling and dropout resulting in focal parenchymal collapse and disorganization of the normal tubulosinusoidal pattern. Ultrastructurally, these apparently irreversibly swollen hepatocytes contained flocculent densities in dispersed, electron lucent cytoplasmic matrixes and cell membranes were often ruptured. In less severely affected hepatocytes, marked diffuse distention of the endoplasmic reticulum was sometimes seen, and foci of cytoplasmic degradation (membrane bound areas containing amorphous cytoplasmic debris) were commonly present, especially in higher dose groups. In addition, throughout the study, occasional hepatocytes contained lipid-like and/or rounded electron dense nuclear inclusions. By 45 and 60 days PE, foci of hepatocellular dropout had resulted in the development of cystic spaces typically subjacent to the space of

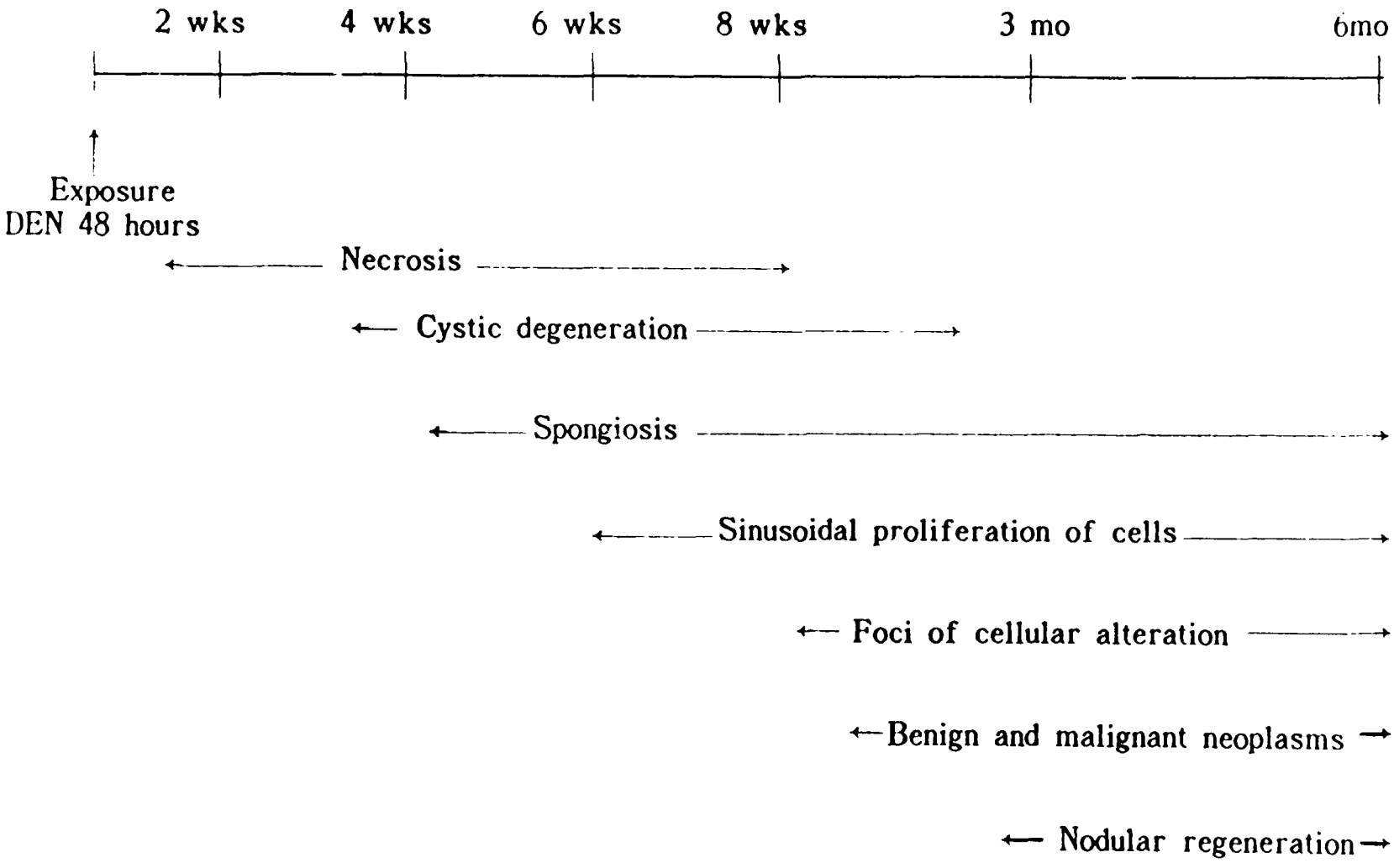
Table I. Degenerative and proliferative hepatic lesions in the medaka at each DEN exposure and sacrifice[1]

Group/ Days PE[2]	Normal liver histology		Individual hepatocellular necrosis		Cellular vacuolation		Globular cytoplasmic inclusions		Spongiosis hepatis	
I-17	4/6	(67)	-	-	1/6	(16)	1/6	(16)	-	-
II-17	5/10	(50)	-	-	2/10	(20)	1/10	(10)	3/10	(30)
III-17	0/5	(0)	2/5	(40)	1/5	(20)	4/5	(80)	-	-
C	10/10	(100)	-	-	-	-	-	-	-	-
I-31	3/10	(30)	1/10	(10)	1/10	(10)	-	-	-	-
II-31	3/10	(30)	1/10	(10)	5/10	(50)	1/10	(10)	2/20	(20)
III-31	0/9	(0)	2/9	(22)	6/9	(67)	1/9	(11)	4/9	(44)
C	10/10	(100)	-	-	-	-	-	-	-	-
I-46	5/10	(50)	2/10	(20)	3/10	(30)	-	-	2/10	(20)
II-46	3/11	(27)	4/11	(36)	3/11	(27)	-	-	3/11	(27)
III-46	0/9	(0)	3/9	(33)	3/9	(33)	1/9	(11)	4/9	(44)
C	10/10	(100)	-	-	-	-	-	-	-	-
I-59	3/10	(30)	3/10	(30)	2/10	(20)	-	-	2/10	(20)
II-59	3/10	(30)	1/10	(10)	5/10	(50)	-	-	5/10	(50)
III-59	1/8	(13)	-	-	5/8	(63)	-	-	5/8	(63)
C	6/6	(100)	-	-	-	-	-	-	-	-
I-94	6/13	(46)	-	-	5/13	(38)	-	-	2/13	(15)
II-94	2/17	(18)	2/17	(12)	6/17	(35)	1/17	(6)	9/17	(53)
III-94	0/10	(0)	-	-	5/10	(50)	4/10	(40)	8/10	(80)
C	5/7	(71)	-	-	-	-	-	-	2/7	(29)
I-191	4/10	(40)	1/10	(10)	5/10	(50)	1/10	(10)	5/10	(50)
II-191	2/62	(3)	1/62	(2)	45/62	(73)	5/62	(8)	27/62	(44)
III-191	0/14	(0)	-	-	10/14	(71)	1/14	(7)	7/14	(50)
C	8/10	(80)	-	-	-	-	-	-	2/10	(20)

[1]Percentages rounded to nearest whole number in parenthesis.

[2]I=100 mg/L; II=200 mg/L; III=400 mg/L; C = 0 mg/L DEN at 17, 31, 46, 59, 94, and 191 days post-exposure.

Table II. Progression of Hepatic Lesions Seen After 48 Hour Exposure to DEN.



Disse which contained ghost remnants of hepatocytes and occasional perisinusoidal cells associated with collagen fibers. Necrosis of associated endothelial lining cells and nearby bile ductular cells seemed in these regions to be secondary to hepatocellular loss. These features combined to result in a marked los in the structural integrity of the sinusoidal wall.

Three changes frequently observed throughout the study were the presence of eosinophilic cytoplasmic inclusions, spongiosis hepatis, and cellular vacuolization. Eosinophilic inclusions were multifocal to diffuse and ultrastructurally were membrane bound cytoplasmic bodies of amorphous content which appeared to coalesce and enlarge on occasion such as to compress the ainder of the cell to a thin peripheral rim. Spongiosis hepatis at early sacrifices appeared similar histologically and ultrastructurally to what has been previously described (8,19). However, at later sacrifices and particularly at higher exposure levels, there was commonly present the proliferation of cells which tended to fill the spaces provided by processes of perisinusoidal cells. Cellular vacuolization was either focal or diffuse, and appeared most commonly to be fatty change; however the composition of this lesion in this study has not yet been determined and thus was given this general designation.

Foci of cellular alteration (FCA) (Table III) were first observed in this study around 60 days PE and were regularly seen thereafter. By 90 and 180 days PE with higher exposure levels they enlarged in some cases to form more appropriately called areas of cellular alteration. One relatively late change seen in the mid and high dose groups was focally extensive vacuolar hepatocellular degeneration and necrosis with regenerative nodules. These were regenerative islands or areas of hepatocytes in compressed degenerative residual parenchyma.

Table III. Foci and areas of cellular alteration, benign neoplasms, and other proliferative lesions in medaka at each DEN exposure and sacrifice[1]

Group/ Days PE	Foci of cellular alteration		Areas of cellular alteration		Adenoma		Cholangioma		Proliferative lesions - uncertain origin[2]	
I-46	-	-	-	-	-	-	-	-	-	-
II-46	-	-	-	-	-	-	-	-	2/11	(18)
III-46	-	-	-	-	-	-	-	-	1/9	(11)
C	-	-	-	-	-	-	-	-	-	-
I-59	-	-	-	-	-	-	-	-	-	-
II-59	1/10	(10)	-	-	-	-	-	-	-	-
III-59	3/8	(38)	-	-	-	-	-	-	1/8	(13)
C	-	-	-	-	-	-	-	-	-	-
I-94	-	-	-	-	-	-	-	-	-	-
II-94	5/17	(29)	1/17	(6)	-	-	-	-	1/17	(6)
III-94	-	-	1/10	(10)	1/10	(10)	-	-	2/10	(20)
C	-	-	-	-	-	-	-	-	-	-
I-191	2/10	(20)	-	-	-	-	-	-	-	-
II-191	9/62	(15)	2/62	(3)	7/62	(11)	2/62	(3)	4/62	(6)
III-191	3/14	(21)	1/14	(7)	-	-	-	-	3/14	(21)
C	-	-	-	-	-	-	-	-	-	-

[1]Percentages rounded to whole numbers in parenthesis.

[2]Proliferation of undetermined cell types in multiple locations - see text.

There was also a relatively common, possibly progressive sinusoidal and perisinusoidal proliferative change of uncertain origin and consequence, seen by 45 days PE (Table III), which consisted histologically of small oval cells with minimal cytoplasm, or rounder larger cells with moderate cytoplasm, or a mixture of both. This change was not readily seen in the low level exposure group. Predominantly oval shaped cells were present in small foci adjacent to and within sinusoids at earlier sacrifices, but mixed populations were in some cases quite extensive and locally invasive with marked cellular and nuclear atypia at later sacrifices replacing large portions of the hepatic parenchyma. These cells appear to represent more than one population of cells. The development of these foci however may be related to the marked loss of integrity of the sinusoidal wall produced by this type of exposure regimen at higher exposure levels, leading to the possible proliferation of multiple affected cell types. There was also a number of benign and malignant neoplasms which are shown in Tables III and IV. Many of these neoplasms were quite variable and complex and often appeared to consist of more than one cell type.

In summary, the exposure of medaka to high levels of DEN for a short period of time resulted in many similarities and some differences in the hepatic lesions produced compared to longer term, lower level exposures. Higher exposure levels resulted in increased incidence and severity of many of the lesions seen, including malignancy, within the time frame of the study.

Table IV. Numbers of carcinomas and sarcomas seen in medaka at each exposure and sacrifice.

	I	II	III	C
Carcinomas				
Hepatocellular (well differentiated)	1 (94 d PE)	1 (191 d PE)	-	-
Hepatocellular (moderately differentiated)	-	1 (191 d PE)	-	-
Cystadenocarcinoma	-	1 (94 d PE)	-	-
Cholangiocarcinoma	-	1 (191 d PE)	1 (191 d PE)	-
Sarcomas				
Hemangiopericytoma	-	-	1 (191 d PE)	-
Hemangioendothelioma	-	-	1 (191 d PE)	-
Mixed carcinoma/sarcoma	-	-	1 (191 d PE)	-
Neoplasms of less certain origin[1]				
Perisinusoidal cell	-	1 (191 d PE)	-	-
Histiocytic cell	-	-	1 (94 d PE) 1 (191 d PE)	-
Undifferentiated	-	1 (46 d PE)	1 (191 d PE)	-
Total[2]	1/59 (1.7%)	6/120 (5.0%)	7/55 (12.7%)	0/53 (0%)

[1]Tentative diagnosis based on ultrastructural and/or histologic features.

[2]Out of samples submitted for histology

C. Results - Part B

1. Morphology

The primary cell types considered were hepatocytes, biliary epithelial cells, endothelial cells, Ito cells, and macrophages. From day zero hatching to eight days post hatch (PH), hepatocytes were characterized primarily by abundant glycogen, mitochondria, and strands of rough endoplasmic reticulum. Lipid droplets become more evident around six days PH. The hepatocytes had polygonal and columnar shapes with a microvillus edge abutting on the sinusoidal side. Binucleate cells were common. From 8-11 days PH, peroxisomes and small lysosomal structures were visible but not abundant. Biliary epithelial cells were seen individually or in clusters as small, dark angular cells with a high nucleus to cytoplasmic ratio. They were joined to each other or to hepatocytes at the canalicular border. Perisinusoidal cells were also small and dark with a very high nucleus to cytoplasmic ratio; angular nuclei with clumped chromatin. At these early ages, occasional desmosomes were seen but lipid was not evident. Few microfilaments could be identified. Cells identified as macrophages were present even at the earliest ages examined and had abundant pale cytoplasm with multiple, irregular lysosomes and prominent golgi bodies. They were located in Disse space or often adjacent to biliary epithelial cells. Endothelial cells were elongated with sparse organelles which lined the sinusoids. Weibel Palade bodies, microtubulated cytoplasmic bodies often used to identify endothelial cells in mammals, were not identified.

By 32 days PH, glycogen was still abundant in hepatocytes, with lipid and lysosomes slightly more prominent than at earlier ages. Other cell types were morphologically similar as described above. An additional cell type was seen, located in Disse space, which was large with numerous cytoplasmic projections but without intercellular junctions, abundant dark cytoplasm, and lysosomes. These cells were not definitely identified but may represent Kupffer cells. They were uncommonly seen.

By 61 days PH, glycogen in hepatocytes was somewhat reduced compared to earlier ages, and cytoplasmic lipid was common. Other cell types were more easily categorized and retained their respective distinguishing features such as cytoplasmic lysosomal abundance in macrophages. Desmosomes and microfilaments were more easily identified in perisinusoidal cells at this age. Biliary epithelial cells were primarily identified based on junctional features with each other and hepatocytes (i.e., the canaliculus).

2. Morphometry

Because of the limited amount of tissue present in each fish at each age, morphometric evaluation was done only at the ultrastructural level. Three age groups were compared; < 11 days PH, 32 days PH, and 61 days PH. The percentages of each cell type and space in the hepatic parenchyma was quantitated as shown in Table V. Included were hepatocytes, biliary epithelial cells (primarily preductular cells), endothelial cells, macrophages, perisinusoidal cells, sinusoids, canaliculi, and disse space. Two additional categories; cells, formed by the addition of point counts from all cells, and space, formed by the addition of all spaces are also included.

Table V. Percentages of each liver cell and space component at each age.

	Groups (age post hatch)		
	< 11 days	32 days	61 days
Cell			
Hepatocytes	88.92[1,2]	92.63	92.42
Biliary epithelial cells	0.39	00.53	0.65
Endothelial cells	1.06[3]	0.66	0.84
Macrophages	0.09	0.05	0.08
Perisinusoidal cell	0.10	0.08[2]	0.18
Total cells	90.55[2,3]	93.92	94.21
Space			
Sinusoid	5.38[1,4]	3.03	3.18
Canaliculi	0.71	0.56	0.51
Disse	2.63	2.21	1.93
Total space	8.72[1,2]	5.81	5.64

[1] $p < .05$ compared to 32 day group

[2] $p < .05$ compared to 61 day group

[3] $p < .10$ compared to 32 day group

[4] $p < .10$ compared to 61 day group

The overall impression from Table V is that at younger ages, the liver consists of more spaces and less cells than at older ages. This is apparently due primarily to a significantly lesser percentage of hepatocytes, and a greater percentage of sinusoidal space at < 11 days of age compared to 32 and 61 days of age. The percentages of canalicular and disse space are also slightly greater in the youngest group, and these differences are also reflected in the space totals. Differences between other cell types between groups were minimal and subject to increased error because of their low percentage contributions. Putative kupffer cells, seen only rarely, were not included in the computations.

Because of the fixative artifacts that would have confounded intracellular data, morphometric evaluation of intracellular organelle composition was not attem .

D. Conclusions

Part A has successfully resulted in providing the following information:

1. The short term exposure regimen used by USABRDL results in many similarities but some differences from studies using lower level, longer term exposures of DEN. However, one criticism leveled against using short term exposure is that there is less variety in types of neoplasms produced. That was not proven to be the case here.
2. There was a direct relationship between exposure level and the incidence and severity of many lesions seen. At the highest level, the increased level of hepatic parenchymal destruction seemed to promote the development of many of the lesions seen.

One portion of Part A that yielded less than optimal results was autoradiography. Occasional labeling was observed but was minimal, although it did indicate that the technique was working to some degree. Limited amounts of hepatic tissue and a midrange exposure level may have contributed to low labeling. A higher exposure level and more frequent early collections may have been more successful.

Part B has successfully resulted in providing the following information.

1. The morphologic appearance of various hepatic cell types did not drastically change with age, but did acquire more "typical" distinguishing characteristics with age.
2. The percentages of each cell type did not change appreciably within the confines of the study with the exception of hepatocytes. With age there was a greater percentage of the parenchyma taken up by hepatocytes at the cost of spaces such as sinusoids. This increase could be due to an increase in hepatocyte numbers and/or size with age.

One problem which arose in Part B was fixation related, artifacticious mitochondrial swelling which did not allow for morphometric evaluation of the cellular composition of individual hepatocytes as too much error would have been introduced. This problem occurred (now recognizable in hindsight) because the entire fish was placed in fixative, and even with the abdomen opened, optimal preservation of tissue did not occur. The problem can be avoided by removing the liver even in very small fish and mincing the tissue.

IV. Future Studies

The continuation of this project will utilize the information obtained and described in this report, and will entail in depth determination of cell types involved in some of these processes using immunohistochemical techniques. This continuation project will yield significant information about cells which proliferate secondary to carcinogenic exposure, and the significance of it when they do. This data is most important because it will increase the predictive ability of the aquatic bioassay.

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ABSTRACTS

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